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Toxicogenomics of A375 human malignant melanoma cells treated with arbutin

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Summary

Although arbutin is a natural product and widely used as an ingredient in skin care products, its effect on the gene expression level of human skin with malignant melanoma cells is rarely reported. We aim to investigate the genotoxic effect of arbutin on the differential gene expression profiling in A375 human malignant melanoma cells through its effect on tumorigenesis and related side-effect. The DNA microarray analysis provided the differential gene expression pattern of arbutin-treated A375 cells with the significant changes of 324 differentially expressed genes, containing 88 up-regulated genes and 236 down-regulated genes. The gene ontology of differentially expressed genes was classified as belonging to cellular component, molecular function and biological process. In addition, four down-regulated genes of AKT1, CLECSF7, FGFR3, and LRP6 served as candidate genes and correlated to suppress the biological processes in the cell cycle of cancer progression and in the downstream signaling pathways of malignancy of melanocytic tumorigenesis.

Introduction

Arbutin, a natural compound of beta-D-glucopyranoside of hydroquinone, is widely used as an ingredient in skin care products [1]. It is effective in the treatment of various cutaneous hyperpigmentations and has the inhibitory effects on the melanogenesis in melanoma cells [2–4]. However, recent findings have raised serious questions of concern regarding both the safety and side-effect of arbutin. Although some inhibitory effects of arbutin on melanogenesis in melanoma cells and its mechanism have been elucidated, the intensive study of its biological effect on human genomics level in the regulation of malignant melanogenesis through the functional effect on carcinogenesis is not clear and rarely reported.

Recently, the high throughput technology of DNA microarray has become important in geneomic research and used to analyze gene expression profiling of human melanomas [5–7]. Since the study of gene expression profiling in human melanoma cells treated with arbutin has never been reported, it would be very challenging to use the DNA microarray in studying the biological effect of arbutin on malignant melanoma cells through its action on anti-cancer property. In this study, we aim to investigate the genotoxic effect of arbutin on the differential gene expression profiling

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in A375 human malignant melanoma cells through its effect on melanocytic tumorigenesis for cancer therapy and other related side-effects. We used the DNA microarray technology and bioinformatic tools to explore the differential gene expression profiling in arbutin-treated A375 cells and to classify the differentially expressed genes by Gene Oncology, which provided more understanding of the genetic basis of metabolic and cellular responses in tumergenesis of human skin cancer. In addition, we compared the biological effects of arbutin and kojic acid on differentially expressed genes in A375 cells and also proposed the hypothetical pathway of arbutin-responsive genes correlated in the suppression of tumorigenesis. These candidate genes may lead to consequently aid for early diagnostic and therapeutic applications.

Materials and methods

Materials

Human malignant melanoma cell line, A375 (CRL-1619), was obtained from the ATCC (Rockville, MD, USA). Dulbecco's Modified Eagle's Medium (DMEM), D-PBS and Trypsin-EDTA were purchased from Atlanta Biologicals (Norcross, GA, USA). Fetal bovine serum, sodium pyruvate, antibiotic-antimycotic and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Arbutin, MTT (methylthiazoletetrazolium) and sodium bicarbonate were purchased from Sigma (St. Louis, MO, USA). RNeasy Mini Kit was purchased from Qiagen (Valencia, CA, USA). Cyanine 3- and 5-labeled CTP were purchased from Perkin-Elemer/NEN Life Science (Boston, MS, USA). RNA 6000 Nano LabChip Kit, Low RNA Input Fluorescent Linear Amplification Kit, Human 1A Oligo Microarray Kit (V2), in situ Hybridization Kit Plus, and Stabilization and Drying Solution were purchased from Agilent Technologies (Palo Alto, CA, USA). All other chemicals were purchased from Sigma (St. Loius, MO, USA).

Cell culture

A375 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate and 1% antibiotic-antimycotic in a humidified incubator with 5% CO_2 and 95% air at 37 °C. Upon confluence, the cells were detached by treatment with 0.05% trypsin and 0.53 mM EDTA. During subculture, the medium was replaced every 2–3 days.

Treatment with arbutin and cell viability test

To perform cell attachment, the cells were seeded at 5.0×10^3 cells/well in 96-well tissue culture-treated plates (NUNC^{TM}, Roskilde, Denmark) in 100 μl of culture media. They were then washed with PBS and were cultured either alone or in the presence of various concentrations of arbutin (0.32, 1.6, 8, 40, 200 and 1000 μg/ml) for 72 h. Arbutin was suspended in 0.01% DMSO, and the control was added with the same concentration of DMSO in order to reduce the variation of cell growth inhibition. Triplicate experiments were done for each pair group of arbutin concentration and control. The cell viability was determined by MTT assay [8]. The percentage of cell growth inhibition was calculated as follows: Inhibition (%) = [1-A_{550} nm (arbutin)/A_{550} nm (control)] $\times$ 100%.

RNA preparation and quantitative measurement

After the cells were treated with 8 μg/ml arbutin for 24 h, RNA was extracted by a modified method using TRIzol combined with the RNeasy Mini Kit. The total RNA was quantified by a UV spectrophotometer and RNA quality was evaluated by capillary electrophoresis on an Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip Kit. For each paired sample, the number of independent paired samples of cultured cells that were treated with arbutin either present or absent was done in triplicate.

RNA amplification and labeling

Targets of cRNA were amplified and fluorescently labeled from 0.5 μg total RNA in each reaction using the Agilent Low RNA Input Fluorescent Linear Amplification kit. For each sample pair, the control sample was labeled with Cy3 and the treated sample was labeled with Cy5. After purification using Qiagen's RNeasy mini-spin

columns, the quantification, quality and size distribution of the labeled cRNA targets were then determined by ultraviolet (UV) spectrophotometry and RNA 6000 Nano LabChip Assay.

Microarray hybridization

Hybridization was performed following the Agilent oligonucleotide microarray hybridization user's manual and Agilent in situ Hybridization Kit Plus. Briefly, 2 μg of the labeled cRNA per channel was mixed with 50 μl 10× control targets and nuclease-free water to a final volume of 240 μl. Each sample tube was added with 10 μl of 25× fragmentation buffer and was incubated at 60 °C in a water bath in the dark for 30 min. The reaction was then terminated by adding 250 μl of 2× hybridization buffer. A volume of 500 μl of hybridization mix was applied to Agilent's Human 1A Oligonucleotide Microarray, containing 20,173 (60 mer) oligonucleotide probes, and hybridized in a hybridization rotation oven at 60 °C for 17 h. The slides which disassembled in 6× SSPE and 0.005% N-Lauroylsarcosine were washed with 6× SSPE, 0.005% N-Lauroylsarcosine for 1 min at room temperature, then with 0.06× SSPE, 0.005% N-Lauroylsarcosine for 1 min and with Stabilization and Drying Solution for 30 s.

Data analysis and bioinformatics

The microarray chip was scanned using an Agilent G2565BA Microarray Scanner System, and the Agilent Feature Extraction software 7.5 used defaults for all parameters including a parameterized error model to compute the significance (p-values) of log ratios. The image quantities of interest produced by the image analysis methods were the (R, G) fluorescence intensity pairs for each gene on each array probe, where R = red for Cy5 and G = green for Cy3. An 'MA-plot' was used to represent the normalized (R, G) data, where $M = \log R/G$ and $A = \log \sqrt{(R \times G)}$ [9].

For the bioinformatics tools to search gene ontology, we used the combination of databases to gain information on gene name and symbol, subcellular location, family and superfamily classification, chromosome map location, similar gene, molecular function, biochemical function-related protein and references. The gene search programs were used the following sequential order of databases: NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org), TIGR (http://www.tigr.org) and GeneCards (http://www.genecards.org). In addition, the category classification of gene expression was done by in-house Bulk Gene Search System for Java (BGSSJ) program that is a searching system accomplished by open database connectivity, UniGene database and Gene Ontology knowledgebase, and is available at http://servx8.sinica.edu.tw/bgss-cgi-bin/gene.pl. On the other hand, the protein search program used the Swiss-Prot/TrEMBL (http://www.expasy.ch/sprot), Proteome (http://www.proteome.com/databases/HumanPD/reports) and PubMed (http://www.ncbi.nl.nih.gov/PubMed). Moreover, the combining pathway databases of Biocarta (http://www.biocarta.com), KEGG (http://www.genome.ad.jp/kegg/pathway.html) and the PubMed literature were used to search the correlated signaling pathways and mechanisms in the malignant melanogenesis of A375 cells.

Real-time quantitative PCR (RT-qPCR)

Specific oligonucleotide primer pairs were designed using the analysis Beacon designer 4.00 (Premier Biosoft International) and were then used for real-time quantitative PCR. The sequences of the primers used are: (1) AKT1 (NM_005163, 150 bp): forward 5′-ACCATCACACCACCTGACCAAG-3′ and reverse 5′-CGCCTCTCCATCCCTCCAAG-3′; (2) CLECSF7 (NM_130441, 184 bp): forward 5′-ATCAACACCAGGGAAGAACAGG-3′ and reverse 5′-TCGCACAACGCTCAT CAAGG-3′; (3) FGFR3 (NM_000142, 102 bp): forward 5′-CTGCCAGCCGAGGAGGAG-3′ and reverse 5′-CACCACCAGGATGAACAGG AAG-3′; (4) LRP6 (NM_002336, 183 bp): forward 5′-AGAATGACCTCAGTGGCAACAG-3′ and reverse 5′-GGGTGGCGGTGGGTA GAG-3′. The specificity of each primer pair was tested by using a common reference RNA (Stratagene) to perform a RT-PCR reaction, followed by DNA 500 chip run on Bioanalyzer 2100 (Agilent Technologies) to check the size of the PCR product. Primer pairs of production predicted product size and no other side-product were chosen to conduct the following SYBR reaction.

Real-time quantitative PCR (RT-qPCR) was performed on the LightCycler instrument 1.5 using LightCycler® FastStart DNA Master^{PLUS} SYBR

Green I kit (Roche Applied Science). Briefly, 10 μl reactions, containing 2 μl Master Mix, 2 μl of 0.25 μM gene specific primer and 6 μl of cDNA, were generated as described above. Each sample, which consisted of cDNA generated from all the samples as detailed above, was run in duplicate. The PCR parameters were 95 °C for 10 s, 45 cycles of 95 °C for 10 s, 60 °C for 3 s and 72 °C for 10 s. At the end of the program a melt curve analysis was made, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler software calculated the threshold cycle (Cp), defined as the fractional cycle number at which the fluorescence reached the baseline. The fold expression or repression of the target gene relative to β-actin in each sample was then calculated by the formula:

$$2^{-\Delta\Delta Cp} \text{ where } \Delta\Delta Cp = \Delta Cp_{\text{arbutin sample}} - \Delta Cp_{\text{control sample}},$$

$$\Delta Cp_{\text{arbutin sample}} = Cp_{\text{arbutin-stimulated target gene}} - Cp_{\text{arbutin-stimulated } \beta\text{-actin}},$$

$$\Delta Cp_{\text{control sample}} = Cp_{\text{control target gene}} - Cp_{\text{control } \beta\text{-actin}}$$

Results

Inhibition effect of arbutin on A375 cells

The cell growth of A375 cells was directly inhibited by the increase of arbutin concentrations. After 72 h, the highest concentration of 1000 μg/ml arbutin allowed up to 40% inhibition of A375 cell growth, while the lower concentrations of 0.32, 1.6, 8 and 40 μg/ml arbutin allowed the inhibition of cell growth by only less than 20% (Figure 1). It is indicated that the A375 cell growth was not strongly affected by all arbutin concentrations. In addition, there were no morphological changes in the cells treated with 0.32–40 μg/ml arbutin. According to the safety recommendation of 1% prescription of drug in human skin care product [10], the concentration of 8 μg/ml arbutin was appropriated in the recommended concentration. Also, there was no morphological change in the cells within 24 h in the presence of 8 μg/ml arbutin and the inhibition of cell growth was less than 10%. However, it has never been reported the biological effect of arbutin on the gene expression profiling in A375 cells and other genotoxic side-effects. Thus, it is very interesting to study the genotoxic effect of arbutin on human skin and on the tumorigenesis that are become our purposes of this study.

Microarray analysis of differential gene expression in A375 cells after arbutin treatment

The microarray results showed different fluorescence intensities between Cy3 (control) and Cy5 (arbutin-treated A375 cells), corresponding to the differential expression level of thousands of genes, and represented the different Cy3 and Cy5 signal intensities with a p-value of less than 0.01 ($p < 0.01$) that were considered to be significantly different (Figure 2). An analysis of gene expression changes with human oligonucleotide array revealed that the total number of 357 differentially expressed probes was matched with approximately 324 listed genes, containing 88 up-regulated genes (Table 1) and 236 down-regulated genes (Table 2). In addition, we used the in-house BGSSJ program to search the gene ontology that classified the gene category, according to cellular component, molecular function and biological process. The summary of the category classification of differentially expressed genes in arbutin-treated A375 cells is shown in Figure 3. The total numbers of 75 up-regulated genes and 123 down-regulated genes were classified into each category with different ratios. Some of genes could be classified into more than one category, according to their cellular component, molecular function and biological process. The expressed genes were found to be located in the subcellular region rather than organelle, extracellular region, protein complex and extracellular matrix, respectively. It may indicate the signal transduction pathway occurring from extracellular region to intracellular region because some of genes served as receptors and/or transporters and have mediated the signaling genes

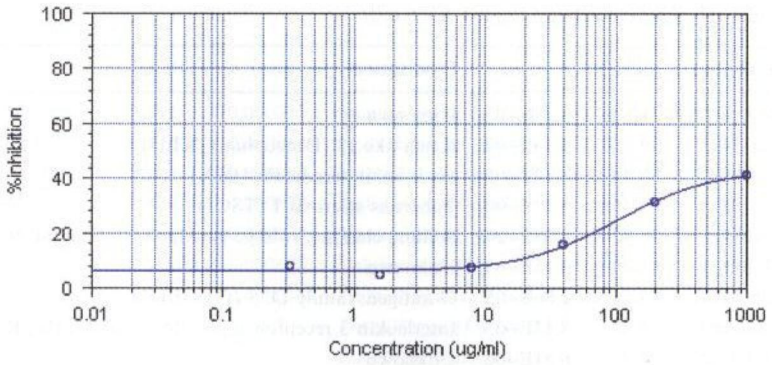


Figure 1. Effect of arbutin concentrations on the growth inhibition of A375 cells at 72 h. The concentrations of arbutin used were 0.32, 1.6, 8, 40, 200 and 1000 μg/ml.

to another region. So, it is possible to be found in both regions. Otherwise, they possessed the molecular functions in different ratios of binding, catalytic activity, signal transducer activity, transcription regulator activity, enzyme regulator activity, transporter activity, structural molecule activity and translation regulator activity. Moreover, the expressed genes involved some biological processes, such as cellular process, physiological process, development and regulation of biological process. Thus, this category classification provided more useful information of gene ontology in response to arbutin treatment. Furthermore, we suggest that all of the differentially expressed genes may be regulated by various gene networks and possess related molecular functions involving biological processes in the regulation of malignant tumorigenesis.

RT-qPCR validation of array analysis

The arbutin-responsive genes in A375 cells chosen for RT-qPCR analysis were selected from the order of p values that were determined by microarray analysis, and from the database search of biological functions correlated with carcinogenesis. The selected genes were validated by RT-qPCR analysis to confirm the result of gene expression level with microarray data and the result of gene expression level of four expressed

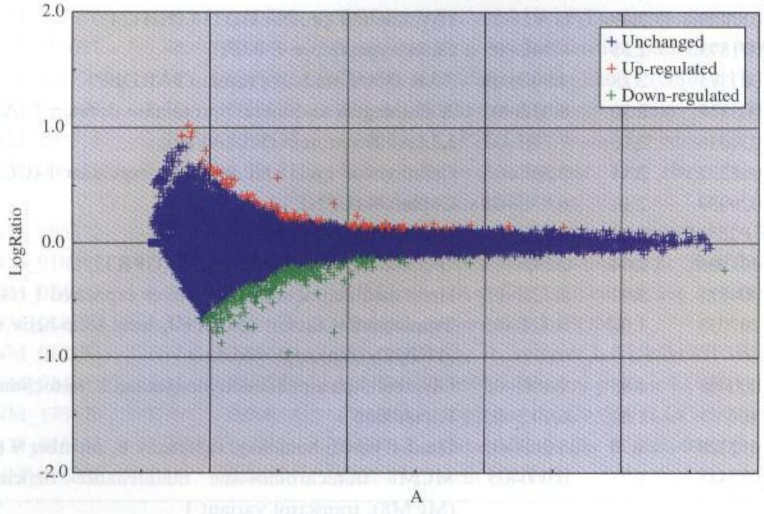


Figure 2. MA-plot of microarray data represents the differentially expressed probes of arbutin-treated A375 cells. A representative probe of comparative experiments (normal mRNA labeled with Cy3 and arbutin-treated mRNA labeled with Cy5) where R = log (Cy5/Cy3) represents the common log ratio of the two dyes and A = [log (Cy5) + log (Cy3)]/2 is the average logarithmic fluorescence intensities of both channels. The gene expression pattern shows approximately 357 significantly differentially expressed probes ($p < 0.01$). Blue + represents any data point whose log ratio is not significantly different from 0; red + and green + represent data points whose log ratios are greater or less than 0, respectively ($p < 0.01$).

Table 1. Up-regulated genes (88 genes) in arbutin-treated A375 cells.

Gene name	Accession no.	Ratio	*p* Value	Description
A_23_P170387	A_23_P170387	10.41	3.28E-07	Unknown
KLHL13	NM_033495	9.31	4.34E-07	Kelch-like 13 (Drosophila) (KLHL13)
EPX	NM_000502	8.42	3.14E-05	Eosinophil peroxidase (EPX)
TSC1	NM_000368	8.31	3.02E-06	Tuberous sclerosis 1 (TSC1)
SCN1B	NM_001037	6.32	8.38E-07	Sodium channel, voltage-gated, type I, beta (SCN1B), transcript variant a
A_23_P86598	A_23_P86598	5.20	3.15E-05	Unknown
GAGED3	NM_130777	4.54	1.86E-05	G antigen, family D, 3 (GAGED3)
IL3RA	NM_002183	4.50	5.11E-05	Interleukin 3 receptor, alpha (low affinity) (IL3RA)
A_23_P32966	A_23_P32966	4.31	6.51E-06	Unknown
FBXO16	NM_172366	4.22	2.94E-05	F-box only protein 16 (FBXO16)
VPS39	NM_015289	4.10	7.83E-06	Vacuolar protein sorting 39 (yeast) (VPS39)
PARG	NM_003631	4.05	2.23E-05	Poly (ADP-ribose) glycohydrolase (PARG)
NRP2	NM_201266	3.94	5.99E-05	Neuropilin 2 (NRP2), transcript variant 1, mRNA
ANKRD23	NM_144994	3.65	2.80E-06	Ankyrin repeat domain 23 (ANKRD23)
FLJ13611	NM_024941	3.61	1.22E-05	Hypothetical protein FLJ13611 (FLJ13611)
THC1991976	THC1991976	3.55	3.44E-05	AF244540 immunodominant membrane protein precursor {Aster yellows phytoplasma}
AEBP2	NM_153207	3.54	1.63E-06	AE binding protein 2 (AEBP2)
ZBTB20	NM_015642	3.36	4.44E-05	Zinc finger protein 288 (ZNF288)
NR2F1	NM_005654	3.27	1.43E-05	Nuclear receptor subfamily 2, group F, member 1 (NR2F1)
KLRC4	NM_013431	3.24	6.16E-05	Killer cell lectin-like receptor subfamily C, member 4 (KLRC4)
CAPNS2	NM_032330	3.17	8.71E-05	Calpain small subunit 2 (CAPNS2)
APOM	NM_019101	3.12	5.14E-05	Apolipoprotein M (APOM)
SLC23A3	NM_144712	2.65	5.34E-06	Solute carrier family 23 (nucleobase transporters), member 3 (SLC23A3)
A_23_P10060	A_23_P10060	2.62	2.04E-05	Unknown
C18orf11	NM_022751	2.62	7.04E-05	Chromosome 18 open reading frame 11 (C18orf11)
DDEF1	NM_018482	2.58	3.21E-05	Development and differentiation enhancing factor 1 (DDEF1)
HIST1H2AC	NM_003512	2.55	2.23E-05	Histone 1, H2ac (HIST1H2AC)
HH114	NM_032499	2.49	6.79E-05	Hypothetical protein HH114 (HH114)
CPE	NM_001873	2.35	4.34E-05	Carboxypeptidase E (CPE)
TARDBP	NM_007375	2.28	4.80E-05	TAR DNA binding protein (TARDBP)
ADAM7	NM_003817	2.20	8.80E-06	A disintegrin and metalloproteinase domain 7 (ADAM7)
KIAA1706	NM_030636	2.16	7.74E-05	KIAA1706 protein (KIAA1706)
CCAR1	NM_018237	2.11	2.38E-05	Cell division cycle and apoptosis regulator 1 (CCAR1)
CPNE5	NM_020939	2.10	6.83E-05	Copine V (CPNE5)
A_23_P251953	A_23_P251953	2.09	7.22E-05	Unknown
GPR32	NM_001506	2.04	2.59E-05	G protein-coupled receptor 32 (GPR32)
HAND1	NM_004821	2.02	2.72E-05	Heart and neural crest derivatives expressed 1 (HAND1)
TCF12	NM_207038	1.97	6.27E-05	Transcription factor 12 (HTF4, helix-loop-helix transcription factors 4) (TCF12), transcript variant 4
GPD2	NM_000408	1.85	9.10E-05	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial) (GPD2)
RAB3GAP	D31886	1.81	6.14E-05	KIAA0066
DNAJB9	NM_012328	1.76	5.26E-05	DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9)
MCM8	NM_032485	1.73	0.000405	MCM8 minichromosome maintenance deficient 8 (S. cerevisiae) (MCM8), transcript variant 1
BC029424	BC029424	1.71	0.000766	cDNA clone MGC:32677 IMAGE:4285958
FKSG43	NM_032033	1.71	0.000588	FKSG43 gene (FKSG43)
CYP2U1	NM_183075	1.70	0.000978	Cytochrome P450, family 2, subfamily U, polypeptide 1 (CYP2U1)
FLJ40432	NM_152523	1.70	0.000645	Hypothetical protein FLJ40432 (FLJ40432)

Table 1. Continued.

Gene name	Accession no.	Ratio	*p* Value	Description
SPATA13	NM_153023	1.69	0.000371	Spermatogenesis associated 13 (SPATA13)
ECE1	NM_001397	1.68	0.000863	Endothelin converting enzyme 1 (ECE1)
MGC12466	NM_033213	1.66	0.000982	Hypothetical protein MGC12466 (MGC12466)
NEU4	NM_080741	1.66	0.000833	Sialidase 4 (NEU4)
TES	NM_152829	1.66	0.000333	Testis derived transcript (3 LIM domains) (TES), transcript variant 2
POLE2	NM_002692	1.64	0.000625	Polymerase (DNA directed), epsilon 2 (p59 subunit) (POLE2)
GALC	NM_000153	1.63	0.000618	Galactosylceramidase (Krabbe disease) (GALC)
DLGAP4	NM_014902	1.62	0.0007	Discs, large (Drosophila) homolog-associated protein 4 (DLGAP4), transcript variant 1
INPP4B	NM_003866	1.58	0.000504	Inositol polyphosphate-4-phosphatase, type II, 105kDa (INPP4B)
ELKS	NM_178037	1.55	0.000781	Rab6-interacting protein 2 (ELKS), transcript variant beta
FAM49B	NM_016623	1.49	0.00073	Hypothetical protein BM-009 (BM-009)
MKI67	NM_002417	1.49	0.00069	Antigen identified by monoclonal antibody Ki-67 (MKI67)
RNF134	NM_032154	1.49	0.000814	Ring finger protein 134 (RNF134)
NMI	NM_004688	1.47	0.000352	N-myc (and STAT) interactor (NMI)
EFTUD1	NM_024580	1.43	0.00258	Hypothetical protein FLJ13119 (FLJ13119)
IQCB1	NM_014642	1.43	0.00317	IQ calmodulin-binding motif containing 1 (IQCB1)
UBQLN1	NM_013438	1.43	0.00305	Ubiquilin 1 (UBQLN1), transcript variant 1
ANKRD17	NM_032217	1.41	0.00391	Ankyrin repeat domain 17 (ANKRD17), transcript variant 1
EPS8	NM_004447	1.41	0.00383	Epidermal growth factor receptor pathway substrate 8 (EPS8)
HIST1H1A	NM_005325	1.41	0.00382	Histone 1, H1a (HIST1H1A)
FBXO11	NM_025133	1.40	0.00418	F-box only protein 11 (FBXO11), transcript variant 1
HUMAUANTIG	NM_013285	1.40	0.0048	Nucleolar GTPase (HUMAUANTIG)
RPS6KA3	NM_004586	1.40	0.00514	Ribosomal protein S6 kinase, 90kDa, polypeptide 3 (RPS6KA3)
A_23_P17152	A_23_P17152	1.39	0.00504	Unknown
CCT8	D13627	1.39	0.00404	KIAA0002
DDX46	NM_014829	1.39	0.00499	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46 (DDX46)
HTATSF1	NM_014500	1.39	0.00475	HIV TAT specific factor 1 (HTATSF1)
BNIP2	NM_004330	1.38	0.00782	BCL2/adenovirus E1B 19kDa interacting protein 2 (BNIP2)
CGI-94	NM_016037	1.38	0.0049	Comparative gene identification transcript 94 (CGI-94)
OSBPL11	NM_022776	1.38	0.00687	Oxysterol binding protein-like 11 (OSBPL11)
PITPNB	NM_012399	1.38	0.00591	Phosphotidylinositol transfer protein, beta (PITPNB)
PPHLN1	NM_201515	1.38	0.00613	Periphilin 1 (PPHLN1), transcript variant 2
SPTLC1	NM_006415	1.38	0.00681	Serine palmitoyltransferase, long chain base subunit 1 (SPTLC1), transcript variant 1
BCAS2	NM_005872	1.37	0.0078	Breast carcinoma amplified sequence 2 (BCAS2)
C20orf36	NM_018257	1.37	0.00705	Chromosome 20 open reading frame 36 (C20orf36)
KDELC1	NM_024089	1.37	0.00851	KDEL (Lys-Asp-Glu-Leu) containing 1 (KDELC1)
PGM2	NM_018290	1.37	0.00679	Phosphoglucomutase 2 (PGM2)
SF3B1	NM_012433	1.37	0.0066	Splicing factor 3b, subunit 1, 155kDa (SF3B1)
CRI1	NM_014335	1.36	0.00788	CREBBP/EP300 inhibitory protein 1 (CRI1)
TMEM32	NM_173470	1.36	0.008	Hypothetical protein LOC93380 (LOC93380)
ARL6IP	NM_015161	1.35	0.00971	ADP-ribosylation factor-like 6 interacting protein (ARL6IP)
CGI-49	NM_016002	1.35	0.00979	CGI-49 protein (CGI-49)

genes was agreed with the DNA microarray expression data (Table 3). There were v-akt murine thymoma viral oncogene homolog 1 (AKT1), c-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 7 (CLECSF7) or C-type lectin domain family 4, member C (CLEC4C), fibroblast growth factor receptor 3 (FGFR3) and low density

Table 2. Down-regulated genes (236 genes) in arbutin-treated A375 cells.

Gene name	Accession no.	Ratio	*p* Value	Description
7h3	NM_033025	0.74	0.00989	Homo sapiens hypothetical protein FLJ13511 (7h3)
ATAD3B	NM_031921	0.74	0.00887	ATPase family, AAA domain containing 3B (ATAD3B)
ARFIP2	NM_012402	0.73	0.00693	ADP-ribosylation factor interacting protein 2 (arfaptin 2) (ARFIP2)
ASNA1	NM_004317	0.73	0.00759	ArsA arsenite transporter, ATP-binding, homolog 1 (bacterial) (ASNA1)
MGC1203	NM_024296	0.73	0.00731	Hypothetical protein MGC1203 (MGC1203)
RGS19IP1	NM_005716	0.73	0.0074	Regulator of G-protein signaling 19 interacting protein 1 (RGS19IP1)
PIN1	NM_006221	0.72	0.00526	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 (PIN1)
PSMD13	NM_175932	0.72	0.00461	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13 (PSMD13)
SSP411	NM_022827	0.72	0.00595	Hypothetical protein FLJ21347 (FLJ21347)
CBX6	NM_014292	0.71	0.00474	Chromobox homolog 6 (CBX6)
CD151	NM_004357	0.71	0.00415	CD151 antigen (CD151)
JUN	NM_002228	0.71	0.00434	v-Jun sarcoma virus 17 oncogene homolog (avian) (JUN)
PSMD3	NM_002809	0.71	0.00298	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3 (PSMD3)
ARHGEF2	NM_004723	0.70	0.00218	Rho/Rac guanine nucleotide exchange factor (GEF) 2 (ARHGEF2)
CD2BP2	NM_006110	0.70	0.0022	CD2 antigen (cytoplasmic tail) binding protein 2 (CD2BP2)
MGC5395	NM_024060	0.69	0.00159	Hypothetical protein MGC5395 (MGC5395)
PIGQ	NM_004204	0.69	0.00196	Phosphatidylinositol glycan, class Q (PIGQ), transcript variant 2
DPP7	NM_013379	0.68	0.00152	Dipeptidylpeptidase 7 (DPP7)
SDC4	NM_002999	0.67	0.000717	Syndecan 4 (amphiglycan, ryudocan) (SDC4)
UBE2M	NM_003969	0.67	0.000537	Ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast) (UBE2M)
AP1S1	NM_057089	0.66	0.000638	Adaptor-related protein complex 1, sigma 1 subunit (AP1S1)
APTX	NM_175073	0.66	0.000804	Aprataxin (APTX), transcript variant 1, mRNA
ARL2	NM_001667	0.66	0.00061	ADP-ribosylation factor-like 2 (ARL2
Beta4GalNAc-T4	NM_178537	0.64	0.000641	Hypothetical protein FLJ25045 (FLJ25045)
BQ189193	BQ189193	0.64	0.000536	cDNA clone UI-E-EJ1-ajv-o-09-0-UI 5', mRNA
DPYSL2	NM_001386	0.64	0.000382	Dihydropyrimidinase-like 2 (DPYSL2)
FTHFSDC1	NM_015440	0.64	0.000892	Formyltetrahydrofolate synthetase domain containing 1 (FTHFSDC1)
ADRB3	NM_000025	0.63	0.000605	Adrenergic, beta-3-, receptor (ADRB3)
HOXB8	NM_024016	0.63	0.000603	Homeo box B8 (HOXB8)
ILT10	NM_024317	0.63	0.000451	Leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 5 (ILT10)
MAFK	NM_002360	0.63	0.000352	v-Maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian) (MAFK)
SYMPK	NM_004819	0.62	5.93E-05	Symplekin (SYMPK)
A_23_P123389	A_23_P123 389	0.61	0.000899	Unknown
ENST00000329697	ENST00000329697	0.61	0.000304	Xenobiotic/medium-chain fatty acid:CoA ligase
FLJ12242	NM_024681	0.61	0.00089	Hypothetical protein FLJ12242 (FLJ12242)
GPRC5D	NM_018654	0.61	0.000349	G protein-coupled receptor, family C, group 5, member D (GPRC5D)
INA	NM_032727	0.61	0.000181	Internexin neuronal intermediate filament protein, alpha (INA)
MGC33867	BC004501	0.61	5.93E-05	Hypothetical protein MGC33867 (cDNA clone IMAGE:3835289)
PCTK1	NM_033018	0.61	7.91E-05	PCTAIRE protein kinase 1 (PCTK1)
PUNC	NM_004884	0.61	0.000473	Putative neuronal cell adhesion molecule (PUNC)
CREBL1	NM_004381	0.60	4.72E-05	cAMP responsive element binding protein-like 1 (CREBL1)
KLPH	NM_207338	0.60	0.000992	Likely ortholog of mouse klotho lactase-phlorizin hydrolase related protein (KLPH)
NYD-SP29	NM_145172	0.60	0.000941	Testis development protein NYD-SP29 (NYD-SP29)

Table 2. Continued.

Gene name	Accession no.	Ratio	*p* Value	Description
OR8B8	ENST00000 327930	0.60	0.000414	Olfactory receptor 8B8 (Olfactory receptor TPCR85) (Olfactory-like receptor JCG8) [Source:SWISSPROT;Acc:Q15620]
TULP3	AF045583	0.60	0.000618	Tubby like protein 3 (TULP3)
MGC35308	NM_175922	0.59	4.91E−05	Hypothetical protein MGC35308 (MGC35308)
OTX1	NM_014562	0.59	0.000342	Orthodenticle homolog 1 (Drosophila) (OTX1)
SNIP1	NM_024700	0.59	0.000202	Smad nuclear interacting protein (SNIP1)
CEACAM1	NM_001712	0.58	0.000249	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (CEACAM1)
DLX1	NM_178120	0.58	0.000192	Distal-less homeo box 1 (DLX1)
FAM3D	NM_138805	0.58	0.000344	Family with sequence similarity 3, member D (FAM3D)
KIAA1811	NM_032430	0.58	0.000299	KIAA1811 protein (KIAA1811)
MIDN	AK075506	0.58	7.37E−05	cDNA PSEC0204 fis, clone HEMBA1002706.
MS4A6E	NM_139249	0.58	0.000482	Membrane-spanning 4-domains, subfamily A, member 6E (MS4A6E)
SFXN2	NM_178858	0.58	0.00037	Sideroflexin 2 (SFXN2)
UPLC1	NM_017707	0.58	0.000303	Up-regulated in liver cancer 1 (UPLC1)
ZFP67	NM_015872	0.58	0.000314	Zinc finger protein 67 homolog (mouse) (ZFP67)
GPR26	NM_153442	0.57	8.11E−05	G protein-coupled receptor 26 (GPR26)
KIAA0669	NM_014779	0.57	0.000263	KIAA0669 gene product (KIAA0669)
LOC83468	NM_031302	0.57	0.000162	Gycosyltransferase (LOC83468)
MAPK8	NM_002750	0.57	0.000385	Mitogen-activated protein kinase 8 (MAPK8)
SERPINA5	NM_000624	0.57	2.64E−05	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5 (SERPINA5)
TREH	NM_007180	0.57	0.000633	Trehalase (brush-border membrane glycoprotein) (TREH)
AKT1[a]	NM_005163	0.56	2.14E−05	v-Akt murine thymoma viral oncogene homolog 1 (AKT1)
MGC33407	NM_178525	0.56	6.68E−05	Hypothetical protein MGC33407 (MGC33407)
MVD	NM_002461	0.56	0.000205	Mevalonate (diphospho) decarboxylase (MVD)
PRRX1	NM_006902	0.56	1.66E−05	Paired related homeobox 1 (PRRX1)
RAB6B	NM_016577	0.56	6.40E−05	RAB6B, member RAS oncogene family (RAB6B)
SERPIND1	NM_000185	0.56	9.94E−05	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1 (SERPIND1)
AFAP	NM_021638	0.55	0.000187	Actin filament associated protein (AFAP)
AK026716	AK026716	0.55	6.38E−05	cDNA: FLJ23063 fis, clone LNG04745
ENPP1	NM_006208	0.55	1.98E−05	Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)
FLJ11535	NM_024888	0.55	4.58E−06	Hypothetical protein FLJ11535 (FLJ11535)
PACSIN2	NM_007229	0.55	4.91E−05	Protein kinase C and casein kinase substrate in neurons 2 (PACSIN2)
SLC13A2	NM_003984	0.55	0.000104	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2 (SLC13A2)
TEX13B	NM_031273	0.55	0.000121	Testis expressed sequence 13B (TEX13B)
A_23_P86779	A_23_P86779	0.54	4.10E−05	Unknown
A_23_P9270	A_23_P9270	0.54	1.36E−05	Unknown
AY032661	AY032661	0.54	0.000159	Lung squamous cell carcinoma related protein-2 mRNA
IFIT3	NM_001549	0.54	0.000147	Interferon-induced protein with tetratricopeptide repeats 4 (IFIT4)
IL23A	NM_016584	0.54	2.18E−05	Interleukin 23, alpha subunit p19 (IL23A)
NM_206995	NM_206995	0.54	1.08E−05	Chromosome 10 open reading frame 1 (C10orf1)
PRKX	NM_005044	0.54	0.000123	Protein kinase, X-linked (PRKX)
ACVRL1	NM_000020	0.53	4.56E−07	Activin A receptor type II-like 1 (ACVRL1)
ARX	NM_139058	0.53	1.37E−05	Aristaless related homeobox (ARX)
CLECSF7[a]	NM_130441	0.53	2.09E−05	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 7 (CLECSF7)

Table 2. Continued.

Gene name	Accession no.	Ratio	*p* Value	Description
MDFI	NM_005586	0.53	1.88E−06	MyoD family inhibitor (MDFI)
BST2	NM_004335	0.52	3.58E−06	Bone marrow stromal cell antigen 2 (BST2)
FLJ14442	NM_032785	0.52	1.43E−06	Hypothetical protein FLJ14442 (FLJ14442)
FLJ20245	NM_017723	0.52	6.35E−07	Hypothetical protein FLJ20245 (FLJ20245)
GPR55	NM_005683	0.52	1.23E−05	G protein-coupled receptor 55 (GPR55)
HRMT1L1	NM_001535	0.52	3.06E−05	HMT1 hnRNP methyltransferase-like 1 (S. cerevisiae) (HRMT1L1)
LRP5	NM_002335	0.52	1.73E−05	Low density lipoprotein receptor-related protein 5 (LRP5)
LRP6[a]	NM_002336	0.52	3.36E−06	LRP6
M160	NM_174941	0.52	5.81E−06	Scavenger receptor cysteine-rich type 1 protein M160 (M160)
C19orf33	NM_033520	0.51	7.70E−07	Immortalization-upregulated protein (IMUP)
FRAS1	NM_206841	0.51	2.96E−05	Fraser syndrome 1 (FRAS1)
GMPR	NM_006877	0.51	2.09E−06	Guanosine monophosphate reductase (GMPR)
MGC13275	AK123855	0.51	8.94E−05	cDNA FLJ41861 fis, clone NTONG2008672
PRSS2	NM_002770	0.51	3.41E−05	Protease, serine, 2 (trypsin 2) (PRSS2)
SLCO5A1	NM_030958	0.51	4.78E−05	Solute carrier organic anion transporter family, member 5A1 (SLCO5A1)
A_23_P89499	A_23_P89499	0.50	8.54E−06	Unknown
COH1	NM_015243	0.50	7.38E−05	Cohen syndrome 1 (COH1), transcript variant 3, mRNA
CROC4	AK124566	0.50	2.26E−05	cDNA FLJ42575 fis, clone BRACE3008237.
NCALD	NM_032041	0.50	1.16E−06	Neurocalcin delta (NCALD)
BAG5	NM_004873	0.49	2.31E−07	BCL2-associated athanogene 5 (BAG5)
BCKDK	NM_005881	0.49	1.99E−06	Branched chain alpha-ketoacid dehydrogenase kinase (BCKDK)
BZRAP1	NM_004758	0.49	1.16E−06	Benzodiazapine receptor (peripheral) associated protein 1 (BZRAP1)
FGFR3[a]	NM_000142	0.49	8.82E−07	FGFR3 (achondroplasia, thanatophoric dwarfism) (FGFR3)
KCNAB3	NM_004732	0.49	1.78E−05	Potassium voltage-gated channel, shaker-related subfamily, beta member 3 (KCNAB3)
NKD1	NM_033119	0.49	6.73E−05	Naked cuticle homolog 1 (Drosophila) (NKD1)
NM_017571	NM_017571	0.49	6.95E−05	Hypothetical protein LOC55580 (LOC55580)
OLFML1	NM_198474	0.49	2.32E−06	MVAL564 (UNQ564)
OSBP2	AF288741	0.49	1.29E−07	Oxysterol binding protein 2 (OSBP2)
RASGEF1B	NM_152545	0.49	3.17E−06	RasGEF domain family, member 1B (RASGEF1B)
STARD8	NM_014725	0.49	2.71E−05	START domain containing 8 (STARD8)
BTN2A1	NM_078476	0.48	1.59E−05	Butyrophilin, subfamily 2, member A1 (BTN2A1)
TRAM2	D31762	0.48	9.80E−08	KIAA0057
ZNF497	NM_198458	0.48	5.89E−08	Zinc finger protein 497 (ZNF497)
EFNA3	NM_004952	0.47	1.93E−07	Ephrin-A3 (EFNA3)
SFRP5	NM_003015	0.47	1.28E−05	Secreted frizzled-related protein 5 (SFRP5)
SLC25A2	NM_031947	0.47	1.39E−07	Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 2 (SLC25A2)
WNT10A	NM_025216	0.47	6.74E−09	Wingless-type MMTV integration site family, member 10A (WNT10A)
C19orf4	NM_012109	0.46	1.27E−06	Chromosome 19 open reading frame 4 (C19orf4)
FPGT	NM_003838	0.46	5.79E−06	Fucose-1-phosphate guanylyltransferase (FPGT)
LRRTM4	NM_024993	0.46	2.00E−05	Leucine-rich repeat transmembrane neuronal 4 protein (LRRTM4)
ZNF483	AB075842	0.46	1.37E−05	mRNA for KIAA1962 protein
CD3G	NM_000073	0.45	8.24E−06	CD3G antigen, gamma polypeptide (TiT3 complex) (CD3G)
MGC15716	NM_032370	0.45	1.02E−06	Hypothetical protein MGC15716 (MGC15716)
A_23_P122323	A_23_P122323	0.44	1.85E−06	Unknown
MGC33637	NM_152596	0.44	3.00E−07	Hypothetical protein MGC33637 (MGC33637)
A_23_P395326	A_23_P395326	0.43	8.31E−07	Unknown
COL17A1	NM_000494	0.43	9.87E−05	Collagen, type XVII, alpha 1 (COL17A1)

Table 2. Continued.

Gene name	Accession no.	Ratio	*p* Value	Description
DNAH7	NM_018897	0.43	7.67E−07	Dynein, axonemal, heavy polypeptide 7 (DNAH7)
SLC27A4	AF055899	0.43	6.94E−09	Fatty acid transport protein (FATP)
THC1871477	THC1871477	0.43	4.54E−06	ALU7_HUMAN Alu subfamily SQ sequence contamination warning entry.
AK092138	AK092138	0.42	1.54E−05	cDNA FLJ34819 fis, clone NT2NE2008213
CNKSR2	AF418270	0.42	9.06E−07	Connector enhancer of KSR2B mRNA
ZDHHC2	NM_016353	0.42	2.83E−05	Zinc finger, DHHC domain containing 2 (ZDHHC2)
ZP2	NM_003460	0.42	5.84E−05	Zona pellucida glycoprotein 2 (sperm receptor) (ZP2)
AY360464	AY360464	0.41	8.63E−05	Hypothetical protein LOC146177-like protein mRNA
TGFB3	NM_003239	0.41	2.95E−05	Transforming growth factor, beta 3 (TGFB3)
TLL1	NM_012464	0.41	9.92E−05	Tolloid-like 1 (TLL1)
C1orf1	U88965	0.40	4.34E−07	PO42 gene
FLJ11383	AK021445	0.40	8.08E−05	cDNA FLJ11383 fis, clone HEMBA1000518, weakly similar to PECANEX protein
HPS3	NM_032383	0.40	3.81E−05	Hermansky-Pudlak syndrome 3 (HPS3)
RSBN1	NM_018364	0.40	2.94E−05	Hypothetical protein FLJ11220 (FLJ11220)
CACNG7	NM_031896	0.39	1.27E−07	Calcium channel, voltage-dependent, gamma subunit 7 (CACNG7)
EFS	NM_005864	0.39	2.12E−07	Embryonal Fyn-associated substrate (EFS)
ELA2	NM_001972	0.39	6.18E−07	Elastase 2, neutrophil (ELA2)
LRRC4	NM_022143	0.39	1.43E−06	Leucine rich repeat containing 4 (LRRC4)
MAP4K5	NM_198794	0.39	8.47E−05	Mitogen-activated protein kinase kinase kinase kinase 5 (MAP4K5)
A_23_P202572	A_23_P202572	0.38	3.90E−06	Unknown
EVI2B	NM_006495	0.38	6.35E−05	Ecotropic viral integration site 2B (EVI2B)
FLJ20619	NM_017904	0.38	5.58E−05	Hypothetical protein FLJ20619 (FLJ20619)
LOC51619	NM_015983	0.38	1.20E−06	Ubiquitin-conjugating enzyme HBUCE1 (LOC51619)
UNQ689	AY358528	0.38	1.64E−06	Clone DNA66660 RSTI689 (UNQ689)
USP44	NM_032147	0.38	5.64E−07	Ubiquitin specific protease 44 (USP44)
ADAM19	NM_023038	0.37	1.81E−07	A disintegrin and metalloproteinase domain 19 (meltrin beta) (ADAM19)
BC039714	BC039714	0.37	3.14E−06	cDNA clone MGC:47794 IMAGE:5585073
C1QB	NM_000491	0.37	5.73E−09	Complement component 1, q subcomponent, beta polypeptide (C1QB)
CD160	NM_007053	0.37	1.14E−07	CD160 antigen (CD160)
MGC40179	NM_173472	0.37	8.87E−07	Hypothetical protein MGC40179 (MGC40179)
PDZK10	AB002314	0.37	2.16E−05	mRNA for KIAA0316 protein
A_23_P212149	A_23_P212149	0.36	5.97E−05	Unknown
A_23_P89841	A_23_P89841	0.36	4.64E−06	Unknown
DKFZP761H1710	NM_031297	0.36	3.42E−12	Hypothetical protein DKFZp761H1710 (DKFZP761H1710)
SLC18A2	NM_003054	0.36	9.80E−05	Solute carrier family 18 (vesicular monoamine), member 2 (SLC18A2)
TCN1	NM_001062	0.36	1.98E−08	Transcobalamin I (vitamin B12 binding protein, R binder family) (TCN1)
ZNF354B	NM_058230	0.36	4.88E−06	Zinc finger protein 354B (ZNF354B)
ABCA13	NM_152701	0.35	1.12E−06	ATP binding cassette gene, sub-family A (ABC1), member 13 (ABCA13)
AF090929	AF090929	0.35	2.90E−07	Clone HQ0477 PRO0477p mRNA
BC032472	BC032472	0.35	9.92E−06	Similar to caspase 4, apoptosis-related cysteine protease, clone IMAGE:5216666
MGC34837	NM_152377	0.35	5.69E−05	Hypothetical protein MGC34837 (MGC34837)
TAL1	NM_003189	0.35	3.85E−08	T-cell acute lymphocytic leukemia 1 (TAL1)
TNFSF6	NM_000639	0.35	1.29E−05	Tumor necrosis factor (ligand) superfamily, member 6 (TNFSF6)
D82326	D82326	0.34	7.13E−06	mRNA for Na^+-independent neutral and basic amino acid transporter
TTC18	NM_145170	0.34	8.71E−07	Tetratricopeptide repeat-containing protein (LOC118491)

Table 2. Continued.

Gene name	Accession no.	Ratio	*p* Value	Description
ADAMTS3	NM_014243	0.33	4.34E−06	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 3 (ADAMTS3)
AGPAT3	NM_020132	0.33	1.43E−05	1-Acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT3)
BC063022	BC063022	0.33	3.78E−06	cDNA clone IMAGE:5246259
MFAP4	NM_002404	0.32	4.90E−06	Microfibrillar-associated protein 4 (MFAP4)
DIAPH2	NM_007309	0.31	1.82E−05	Diaphanous homolog 2 (Drosophila) (DIAPH2)
GUCY1A2	NM_000855	0.31	1.81E−06	Guanylate cyclase 1, soluble, alpha 2 (GUCY1A2)
MGC35030	BC042481	0.31	2.82E−08	Hypothetical protein MGC35030 (cDNA clone MGC:35030 IMAGE:5164982)
SIN3B	AB014600	0.31	2.27E−06	mRNA for KIAA0700 protein
SOCS4	NM_080867	0.31	6.50E−05	Suppressor of cytokine signaling 4 (SOCS4)
A_23_P253353	A_23_P253353	0.30	6.70E−11	Unknown
EMX2	NM_004098	0.30	4.25E−05	Empty spiracles homolog 2 (Drosophila) (EMX2)
FLJ22843	AK026254	0.30	6.67E−06	cDNA: FLJ22601 fis, clone HSI04471
GSDM1	NM_178171	0.30	8.77E−09	Gasdermin (GSDM)
HLF	NM_002126	0.30	4.94E−07	Hepatic leukemia factor (HLF)
SAA4	NM_006512	0.30	3.45E−05	Serum amyloid A4, constitutive (SAA4)
SH3GL2	NM_003026	0.30	2.64E−05	SH3-domain GRB2-like 2 (SH3GL2)
ACMSD	NM_138326	0.29	1.65E−06	Aminocarboxymuconate semialdehyde decarboxylase (ACMSD)
ADAM20	NM_003814	0.29	5.31E−06	A disintegrin and metalloproteinase domain 20 (ADAM20)
C15orf26	NM_173528	0.28	7.14E−05	Hypothetical protein FLJ38615 (FLJ38615)
A_23_P29907	A_23_P29907	0.27	6.46E−08	Unknown
AX721299	AX721299	0.27	7.60E−05	Sequence 259 from Patent WO0220754
C6orf97	NM_025059	0.27	9.23E−06	Chromosome 6 open reading frame 97 (C6orf97)
LGALS14	NM_020129	0.27	5.56E−06	Placental protein 13-like protein (PPL13)
SLC15A3	NM_016582	0.27	1.03E−10	Solute carrier family 15, member 3 (SLC15A3)
ABCC13	NM_138726	0.25	7.39E−06	ATP-binding cassette, sub-family C (CFTR/MRP), member 13 (ABCC13)
NM_152768	NM_152768	0.25	2.33E−06	Hypothetical protein FLJ25378 (FLJ25378)
PKHD1	NM_138694	0.25	8.75E−05	Polycystic kidney and hepatic disease 1 (autosomal recessive) (PKHD1)
ABCA10	NM_080282	0.24	2.86E−05	ATP-binding cassette, sub-family A (ABC1), member 10 (ABCA10)
BMPR1B	NM_001203	0.24	3.08E−07	Bone morphogenetic protein receptor, type IB (BMPR1B)
C14orf148	NM_138791	0.24	5.68E−06	Hypothetical protein FLJ32809 (LOC122945)
FGF5	NM_004464	0.24	2.93E−09	Fibroblast growth factor 5 (FGF5)
ADAMTS9	NM_020249	0.23	6.69E−05	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9 (ADAMTS9)
CPB1	NM_001871	0.23	5.82E−09	Carboxypeptidase B1 (tissue) (CPB1)
FMNL3	NM_175736	0.23	1.84E−07	Formin-like 3 (FMNL3)
L17325	L17325	0.23	7.47E−05	Pre-T/NK cell associated protein (1D12A2)
SEMG1	NM_003007	0.23	4.55E−05	Semenogelin I (SEMG1)
SLC7A3	NM_032803	0.23	2.09E−05	Solute carrier family 7 (cationic amino acid transporter, y + system), member 3 (SLC7A3)
ZNF141	NM_003441	0.23	2.11E−05	Zinc finger protein 141 (clone pHZ-44) (ZNF141)
DMXL1	NM_005509	0.22	1.45E−07	Dmx-like 1 (DMXL1)
GPC3	NM_004484	0.22	3.05E−06	Glypican 3 (GPC3)
NEGR1	NM_173808	0.22	5.06E−05	Neuronal growth regulator 1 (NEGR1)
PDZK1	NM_002614	0.22	2.16E−05	PDZ domain containing 1 (PDZK1)
RFPL2	NM_006605	0.22	8.26E−05	Ret finger protein-like 2 (RFPL2)
ZNF167	NM_018651	0.22	3.58E−05	Zinc finger protein 167 (ZNF167)
MYO3B	NM_138995	0.21	3.06E−07	Myosin IIIB (MYO3B)

Table 2. Continued.

Gene name	Accession no.	Ratio	*p* Value	Description
AK025116	AK025116	0.20	9.77E−07	cDNA: FLJ21463 fis, clone COL04765
C20orf17	NM_173485	0.20	4.87E−05	Chromosome 20 open reading frame 17 (C20orf17)
CASP8	NM_033357	0.20	1.57E−05	Caspase 8, apoptosis-related cysteine protease (CASP8)
GPR155	NM_152529	0.20	1.42E−09	G protein-coupled receptor 155 (GPR155)
NTF5	NM_006179	0.20	2.06E−06	Neurotrophin 5 (neurotrophin 4/5) (NTF5)
RFPL3	NM_006604	0.20	2.77E−07	Ret finger protein-like 3 (RFPL3)
LOC162967	NM_207333	0.19	1.57E−12	Hypothetical protein LOC162967 (LOC162967)
IL6ST	NM_002184	0.18	1.50E−07	Interleukin 6 signal transducer (gp130, oncostatin M receptor) (IL6ST)
PF4	NM_002619	0.18	7.15E−14	Platelet factor 4 (chemokine (C-X-C motif) ligand 4) (PF4)
HSD3B1	NM_000862	0.17	3.81E−07	Hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1 (HSD3B1)
THC1910111	THC1910111	0.17	6.32E−09	BC022679 D13Ertd275e protein {Mus musculus}
F13B	NM_001994	0.16	3.00E−08	Coagulation factor XIII, B polypeptide (F13B)
ZNF41	NM_153380	0.13	9.11E−11	Zinc finger protein 41 (ZNF41)

[a]The down-regulated genes were significantly expressed in agreement with RT-qPCR result.

lipoprotein receptor-related protein 6 (LRP6). They were down-regulated in arbutin-treated cells and have been found to be the tumor suppressor genes in the regulation of carcinogenesis mechanism. In addition, four differentially expressed genes were classified by BGSSJ program and showed the localization in subcellular region, except that CLECSF7 was not found in the classification. AKT1 and FGFR3 were found to be located in the organelle and extracellular regions, respectively. The molecular functions of those genes were involved in binding, catalytic activity and signal transducer activity, and their biological processes were involved in physiological process, cellular process, regulation of biological process and development.

Correlation of differentially expressed genes with signaling pathways of malignant melanogenesis and tumerigenesis

Combining the pathway databases of BioCarta, KEGG and the PubMed literatures, we built the hypothetical model of signaling pathway in the cell cycle and anti-apoptosis in tumorigenesis, in which the differentially expressed genes of AKT1, FGFR3 and LRP6 in arbutin-treated A375 cells are correlated in this pathway (Figure 4). Our hypothetical model suggests that the arbutin-responsive genes of AKT1, FGFR3 and LRP6 are correlated with the AKT signaling pathway, RAS, MAPK and WNT signaling pathways, respectively. FGFR3 and LRP6 normally activated in the membrane region and mediated the correlated genes to the downstream pathways of RAS, MAPK and AKT. The down-regulation of these genes may lead to the suppression of cell proliferation, survival, differentiation and apoptosis of the arbutin-treated A375 cells. Therefore, AKT1, FGFR3 and LRP6 play a key role in the regulation of both malignant melanognesis and tumorigenesis. They may become useful candidate markers for early diagnostic and therapeutic applications of melanoma carcinogenesis.

Discussion

For the cell treatment, we examined the effect of arbutin concentrations on the growth inhibition of A375 cells and those concentrations did not strongly inhibit cell growth even though the incubation time was up to 72 h. We used the concentration of 8 μg/ml arbutin because (i) this was the same concentration as our previous study of kojic acid [11], in which this concentration (8 μg/ml or 0.8%w/v) was less than the saftety recommendation of 1% prescription in human skin care product [10], (ii) this concentration inhibited cell growth with an inhibition of less than 10% and no morpholical change of cells, and (iii) it is avoid the differential gene expression data resulting from cell death response of the

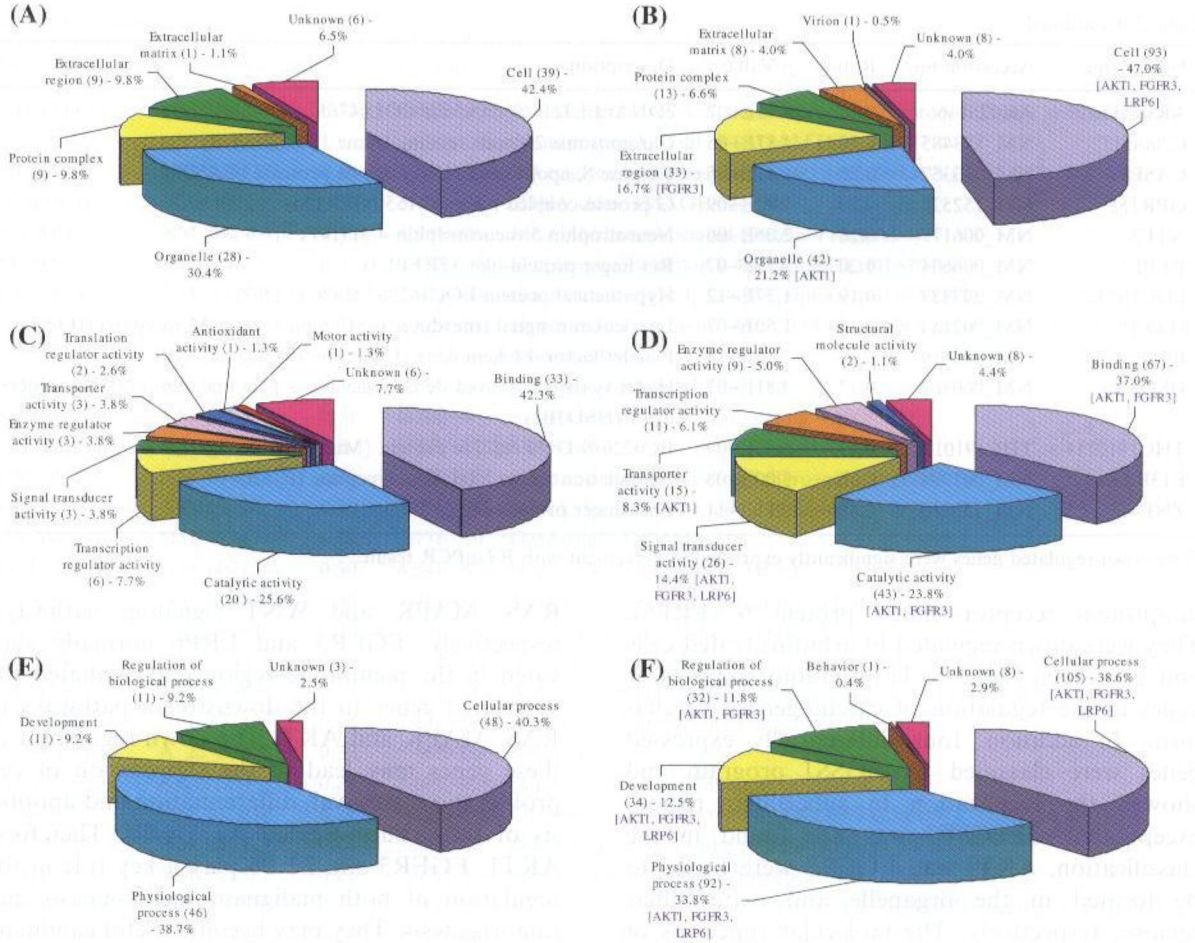


Figure 3. Category classification of up- and down-regulated genes in arbutin-treated A375 cells using the in-house BGSSJ program. The up- and down-regulated genes are classified according to cellular component (A, B), molecular function (C, D), and biological process (E, F), respectively. The following numbers are the classified genes and the percentage of genes in each category. Three down-regulated genes of AKT1, FGFR3 and LRP6 can be classified into each category.

cytotoxicity of a high concentration of arbutin. Under this consideration, the concentration of 8 μg/ml arbutin that is safe for use on human skin was used to study the genotoxic effect on gene expression profiling in A375 cells for examining the differential gene expression and another side-effect through the signaling pathways for cancer therapy. In order to study the effect of arbutin on the gene expression level of A375 cells, the time point of 24 h after arbutin treatment was

Table 3. Comparison of gene expression level of four differentially expressed genes in arbutin-treated A375 cells by microarray and RT-qPCR analysis.

Gene name	Accessions	Description	*p* Value	Microarray	RT-qPCR
1. AKT1	NM_005163	v-Akt murine thymoma viral oncogene homolog 1	2.14E−405	0.56	0.63
2. CLECSF7	NM_130441	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 7, transcript variant 1	2.09E−05	0.53	0.52
3. FGFR3	NM_000142	FGFR3 (achondroplasia, thanatophoric dwarfism), transcript variant 1	8.82E−07	0.49	0.43
4. LRP6	NM_002336	LRP6	3.36E−06	0.52	0.60

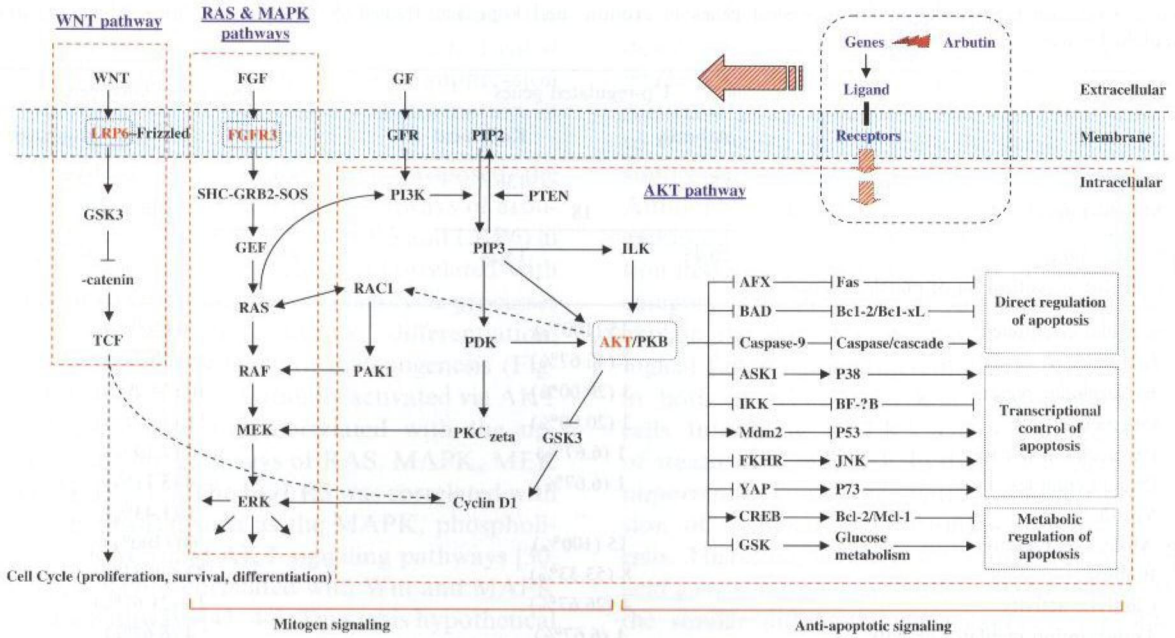


Figure 4. Hypothetical pathways of arbutin-responsive genes (AKT1, FGFR3 and LRP6) in A375 cells correlated with AKT, WNT, RAS and MAPK major signaling pathways in malignant melanogenesis and tumerigenesis. Abbreviations: FGF, fibroblast growth factor; GEF, guanine nucleotide exchange factor; GF, growth factor; GFR, growth factor receptor; GRB2, growth factor receptor-bound protein 2; GSK3, glycogen synthase kinase 3; ILK, integrin-linked kinase; MAPK, mitogen-activated protein kinase 3; PI3K, phosphoinoside-3 kinase; PIP2, phosphatidylinositol-4,5-biphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PDK-1, phosphainositide-dependent kinase-1; PKC, protein kinase C; PTEN, phosphatase and tensin homolog; SON, son of sevenless homolog 1; TCF, transcription factor 7.

chosen for isolating the RNA from cells and studying the gene expression level using microarray analysis.

The changes of differentially expressed genes, which were validated by RT-qPCR analysis, gave the confirmatory quantitative results under stringent condition and revealed that the quantity or expression level of arbutin-responsive genes agreed with the DNA microarray data. It indicated that the validated genes in the robust biomarkers lists obtained precise data. Meanwhile, four significantly arbutin-responsive genes were found to be down-regulated in arbutin-treated A375 cells, and most of the genes are highly effective to the regulation of malignant melanogenesis and/or tumorigenesis process.

AKT1 (v-akt murine thymoma viral oncogene homolog 1 or serine/threonine protein kinase PKB) is frequently up-regulated in human tumors and has been shown to accelerate cell proliferation and to suppress programmed cell death. [12–14]. The gene ontology of its biological process involved in the protein amino acid phosphorylation, nitric oxide biosynthesis, anti-apoptosis, signal transduction and G-protein coupled receptor protein signaling pathway. Its activation via the AKT/PKB signaling pathway is triggered through the engagement of receptor tyrosine kinases by peptide growth factors and cytokines, and possesses a vital role in human malignancy tumorigenesis [15–17]. Consequently, the down-regulated AKT1 gene in arbutin-treated A375 cells may suppress the tumor progression of malignant transformation in melanoma cells [18–20]. Likewise, AKT1 has also been classified as a tumor suppressor gene in a wide variety of tumor cells, such as breast cancer cells, nonsmall cell lung cancer cells and pancreatic adenocarcinoma, and its down-regulation was shown to suppress the malignant progression of tumorigenesis [21–23].

C-type lectin, superfamily member 7 (CLECSF7) is a novel C-type lectin gene that maps close to the natural killer gene complex on human chromosome located at 12p13.2-p12.3 [24]. The protein encoding CLECSF7 gene, called C-type lectin domain family 4 member C (CLEC4C), is the membrane protein and involved in an oligosaccharide-recognition mechanism [25]. This

Table 4. Comparison of differentially expressed genes in arbutin- and kojic acid-treated A375 cells and category classification of matched genes.

Total	Up-regulated genes		Down-regulated genes	
	Arbutin	Kojic acid	Arbutin	Kojic acid
1. Number	88	136	236	225
2. Matched genes	18		98	
3. % Matching	20.45	13.24	41.53	43.56
4. Category classification of matched genes				
(a) Cellular component	15 (100%)		70 (100%)	
Cell	7 (46.67%)		39 (55.71%)	
Extracellular region	3 (20.00%)		10 (14.29%)	
Organelle	3 (20.00%)		11 (15.71%)	
Extracellular matrix	1 (6.67%)		5 (7.14%)	
Protein complex	1 (6.67%)		4 (5.71%)	
Virion	–		1 (1.43%)	
(b) Molecular function	15 (100%)		60 (100%)	
Binding	8 (53.33%)		23 (38.33%)	
Catalytic activity	4 (26.67%)		13 (21.67%)	
Transcription regulator activity	1 (6.67%)		4 (6.67%)	
Transporter activity	1 (6.67%)		3 (5.00%)	
Signal transducer activity	–		13 (21.67%)	
Structural molecule activity	1 (6.67%)		1 (1.67%)	
Enzyme regulator activity	–		3 (5.00%)	
(c) Biological function	21 (100%)		101 (100%)	
Physiological process	9 (42.86%)		33 (32.67%)	
Cellular process	9 (42.86%)		43 (42.57%)	
Regulation of biological process	1 (4.76%)		12 (11.88%)	
Development	2 (9.52%)		13 (12.87%)	

protein could interact with blood DC antigen type-2 (BDCA-2) receptor and involve in the target ligand into antigen processing and peptide loading compartments for presentation to T cells [26], in which its activation mediate induction of IFN-alpha/beta expression in plasmacytoid dendritic cells in the downstream pathway of apoptosis [27–29]. The down-regulated CLECSF7 gene in arbutin-treated A375 cells may be effective to the immune system and may inhibit the apoptosis of melanogenesis.

FGFR3 is one of the four high affinity tyrosine kinase receptors for the FGF family of ligands. On ligand stimulation, it undergoes dimerization and tyrosine autophosphorylation that result in cell proliferation or differentiation, depending on the cell context [30, 31]. The extracellular portion of the protein encoded by FGFR3 gene interacts with fibroblast growth factors, sets in motion an acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance [32, 33]. The inhibition of FGFR3 also induced differentiation and apoptosis in multiple myeloma and is treated as an oncogene that contributes to tumor progression in multiple myeloma including human breast cancer [34–36]. Therefore, the down-regulated FGFR3 gene may suppress the tumor progression of malignant transformation in melanoma cells.

LRP6 is a member of the expanding LDL receptor family and functions as an indispensable co-receptor for the Wnt/beta catenin signaling pathway [37–39]. It has been found to be a candidate oncogenes that promoted cancer cell proliferation and tumorigenesis by altering β-catenin subcellular distribution. In the absence or the deactivating of Wnts, β-catenin is phosphorylated by multiprotein complex, and leads to

suppress the tumorigenesis [40–43]. Thus, the down-regulated LRP6 gene in arbutin-treated A375 cells may be a key mediator in suppression of tumorigenesis of malignant transformation in melanoma cells.

According to our finding, we proposed the hypothetical model of signaling pathways of arbutin-responsive genes (AKT1, FGFR3 and LRP6) in A375 melanoma cells which were correlated with the major signaling pathways in cell cycle processes including proliferation, survival, differentiation, apoptosis and malignancy of melanogenesis (Figure 4). The AKT1 was normally activated via AKT signaling pathway that correlated with the upstream signaling pathways of RAS, MAPK, MEK and ERK [15–17]. The FGFR3 was correlated with several pathways, such as the MAPK, phospholipase Cγ, RAC1 and AKT signaling pathways [30, 31]. The LRP6 is correlated with Wnt and MAPK signaling pathways [41–44]. Thus, this hypothetical model of signaling pathways is a simplified model which can represent the correlation of arbutin-responsive genes with other pathways and provides a clearer understanding of the arbutin-suppression effect on malignancy of melanocytic tumorigenesis. In addition, the CLECSF7 gene was not correlated with this hypothetical model but the deactivated CLECSF7 gene may correlate with another signaling pathway, occurring apoptosis and tumor suppression. Therefore, four down-regulated genes served as key mediators in the cell cycle's signaling pathways and possessed the biological function in the suppression of malignant transformation in arbutin-treated A375 cells.

Moreover, according to our previous study of A375 cells treated with kojic acid [11], we also compared the biological effects of arbutin and kojic acid on the gene expression in A375 cells, in which the same conditions of concentrations, cell treatment and microarray were used. It is the first study of comparative genotoxic effects between arbutin and kojic acid. The purpose of this comparison is to investigate the correlated genotoxic effects on gene expression profiling through the biological function of matched genes in the suppression of tumorigenesis. The comparison of differentially expressed genes between arbutin-and kojic acid-treated A375 cells showed the matched genes of 18 up-regulated genes and 98 down-regulated genes, including four significantly arbutin-responsive genes (AKT1, CLECSF7, FGFR3 and LRP6) (Table 4). The matched number of down-regulated genes was 5.4-fold of up-regulated genes and the matching percentages of down-regulated genes in arbutin and kojic acid were up to 40%, which may indicate the same direction of suppression of tumorigenesis in A375 cells. Although the comparison of differentially expressed genes according to category classification may not give any significant information, the category classification of matched genes can explain the similar molecular functions and biological functions of differentially expressed genes in both of arbutin-and kojic acid-treated A375 cells. Interesting, FGFR3 gene was found in both of treatments, in which FGFR3 gene may be an important key in the regulation of tumor progression of malignant transformation in melanoma cells. Therefore, we suggest that arbutin and kojic acid gave some biological effect on A375 cells with the similar differential gene expression profiling but the regulation of tumor progression may be regulated by different genes or signaling pathways.

In conclusion, we used the high throughput analysis of DNA microarray and bioinformatic tools for a global analysis of differentially expressed genes in arbutin treated A375 malignant melanoma cells. These genes were classified the gene oncology and led to an exploration of more valuable data in the regulation of melanoma carcinogenesis. In addition, four down-regulated genes served as candidate tumor suppressor genes in A375 cells after arbutin treatment. They may deactivate the regulation of malignant tumorigenesis in human malignant melanoma cells and may become useful markers for further diagnostic and therapeutic applications. Moreover, the genotoxic effect of arbutin on gene expression profiling of A375 cells was similar to kojic acid effect, which may indicate the similar regulation of malignant tumorigenesis. However, we will conduct further studies on the effects of arbutin on the biological and molecular mechanisms of human skin cells, and also examine other biological characterizations of arbutin for therapeutic applications.

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